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A Clinical Evaluation of a New Method for HBV DNA Quantitation in Patients With Chronic Hepatitis B

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Selection of HBsAg-positive patients for antiviral therapy requires an estimation of disease activity and viral replication. Serum transaminases and histological analysis are commonly used to assess disease activity, and viral replication is assessed by serological testing of HBeAg and serum hepatitis B virus (HBV) DNA. Dot blot hybridisation may be insufficiently sensitive to corroborate low-grade replication in patients with active hepatitis, and polymerase chain reaction (PCR) may be testing too sensitive for this role. Theoretically an assay of intermediate sensitivity is therefore required. Our aim was to evaluate whether the branched chain DNA (bDNA) assay would fulfil this function. Seventy-one HBsAg-positive patients were tested for HBV DNA by the bDNA assay; 64 were also tested by dot blot hybridisation and, when appropriate, also by PCR. Thirty-seven (52%) patients were positive for HBV DNA by the bDNA assay. HBV DNA was detected in the majority (21/28; 75%) of HBeAg-positive patients but also in 14 of 36 (39%) anti-HBe-positive patients. HBV DNA was detected by the bDNA assay in 20 of 48 (42%) patients negative for HBV DNA by dot blot hybridisation assay. All patients positive for HBV DNA by dot blot hybridisation were also positive by the bDNA assay. Sixteen of twenty-five (64%) patients negative for HBV DNA by the bDNA assay were positive for HBV DNA by PCR. The bDNA assay is a sensitive and reliable method for the detection of HBV DNA. As nucleoside analogue therapy becomes more widely available, the assay should provide a useful tool for the selection for and monitoring of patients on antiviral therapy.

KEY WORDS: chronic hepatitis B, HBV DNA, dot blot, PCR, HBeAg, anti-HBe

INTRODUCTION

Serum HBV DNA is an index of HBV replication [Bonino et al., 1981; Yokusuko et al., 1985; Brechot et al., 1981], and HBV DNA concentrations are useful in the assessment, management, and antiviral treatment of patients with chronic HBV infection [Kam et al., 1982; Kuhns et al., 1989; Pastore et al., 1992]. However, methods for detecting HBV DNA differ in sensitivity and therefore clinical relevance. HBV DNA measured by conventional hybridisation techniques is lost from the serum in approximately 30% of patients undergoing interferon-α therapy [Korenman et al., 1991]. Losses of both HBV DNA and HBeAg from the serum correlate with clinical and biochemical recovery [Brook et al., 1989]. However, HBV DNA is lost earlier than HBeAg and is an important index of response to therapy [Perrillo et al., 1993]. Although HBV DNA can also be detected in HBeAg-negative, anti-HBe-positive patients [Gowans, 1986], dot blot hybridisation is insufficiently sensitive to identify all HBsAg-positive patients with HBV-related liver disease [Monjardino et al., 1991]. The polymerase chain reaction (PCR) is up to 10^4 times more sensitive than conventional hybridisation techniques for the detection of HBV DNA [Kaneko et al., 1989], but its clinical usefulness is limited by its high sensitivity; virtually all HBsAg-positive patients are HBV DNA positive by PCR. Also, patients who lose HBsAg after acute hepatitis B or after interferon-α therapy can still remain positive by PCR [Carman et al., 1991]. Although PCR positivity might represent low-level viral replication, and possible infectivity if the liver is used for allografting [Chazouillères et al., 1994], it might not necessarily indicate liver disease in the host [Loriot et al., 1992].

Newer methods of nucleic acid quantification are becoming available, in which signal rather than DNA amplification is employed. These methods avoid the problem of nonspecific probe hybridisation. One such assay is the branched chain DNA assay (Quantiplex; Chiron Corp., Emeryville, CA). This is a chemiluminescence assay that allows rapid quantification of serum HBV DNA. Previous investigations have suggested that it is more sensitive than conventional hybridisation techniques and less sensitive than PCR [Valinluck et al., 1993; Chen et al., 1995]. Theoretically, therefore, an

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assay of this sensitivity could provide useful clinical information for the management of patients with hepatitis B. Our aim was to evaluate the clinical context of this test in a spectrum of patients with chronic HBV infection and to measure its sensitivity in comparison to the dot blot assay and PCR for HBV DNA.

PATIENTS

Sera from 71 HBsAg-positive patients with a median age of 39 years were tested. All patients were known to have chronic hepatitis B. Fifty-two were male and 19 female. Thirty-nine were thought to have had neonatal exposure to hepatitis B, 13 to have acquired the disease by sexual contact, and 2 to have had occupational exposure. Other or unknown risk factors were implicated in the remainder. Twenty-eight patients were HBeAg positive and anti-HBe negative, 36 patients were HBeAg negative and anti-HBe positive, 4 were both HBeAg and anti-HBe positive, and 3 were both HBeAg and anti-HBe negative. In addition, 15 patients were anti-HDV positive, 4 were anti-HCV positive and 4 were HIV positive.

Liver biopsies were available from 42 of 71 (59%) cases. Eight had mild chronic hepatitis, and 33 had chronic active hepatitis, 12 of them having cirrhosis. One patient had inactive cirrhosis, Twenty-one of these patients had received antiviral therapy following liver biopsy but prior to serum HBV DNA testing. One patient had undergone orthotopic liver transplantation, and two had hepatocellular carcinoma.

MATERIALS AND METHODS

Branched Chain DNA Assay

This assay is available commercially as a "sandwich" nucleic acid hybridisation assay for the quantitative detection of HBV DNA (Quantiplex, Chiron Corp., Emeryville, CA). Briefly, 10 μl of each test serum was incubated with 10 μl of a lysis buffer in a microwell plate and incubated for 30 minutes at 63°C to release HBV DNA from the viral particles. Ten microliters of the target probes in a denaturing mix were added, and the plate was incubated again for 30 min at 63°C. This mix contains oligonucleotide probes that hybridise to both sequences on the minus-sense strand of the viral genome and oligonucleotides bound to the walls of the microwells and also a different set of oligonucleotides complimentary to other sequences on the minus-sense strand of the viral genome and to the branched chain DNA amplifier. Ten microliters of a neutralising reagent were added, and the plate was incubated at 63°C for 18 hours to allow hybridisation to occur.

After cooling, the wells were washed, and 40 μl of the amplifier mix were added. This mix contains the branched chain DNA amplifier, which hybridises to the target probes as described above. After incubating for 30 minutes at 53°C, the wells were washed again, and 40 μl of the alkaline phosphatase labelled probes were added. These hybridise to the branched chain DNA amplifier. The plate was incubated for 15 minutes at 53°C, cooled for 10 minutes, and washed again. Next, 30 μl of the dioxetane substrate were added. The plate was incubated for 25 minutes at 37°C in the luminometer, and then the chemiluminescent signal was read. This signal is proportional to the amount of HBV DNA present and can be quantified against the values obtained from a set of accurately quantified cloned HBV DNA standards to give a final DNA measurement. A single assay can quantify 42 samples performed in duplicate.

DNA measurements are given in genome equivalents per milliliter; however, for the purposes of comparison, we have converted this to picograms per milliliter by multiplying by 3.53×10^{-6}.

Dot Blot Hybridisation

DNA was extracted from 50 μl serum with 20μl 10% NP40 and 20 μl 3% β-mercaptoethanol and denatured with 180 μl 1 M NaOH and 90 μl 2 M NaCl. Up to 180 μl of this reaction mix were applied onto a nylon filter for subsequent hybridisation. The filter was washed with 0.5 M Tris HCl, pH 7.5, and 2.5 M NaCl and then air dried, and the DNA was bonded to the filter using UV irradiation. Blots were prehybridised for 4 hours at 68°C in 6× SSC, 10 mM EDTA, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA. Hybridisation with a ^{32}P-labelled cloned HBV DNA probe was performed in the same solution at 68°C overnight. The blots were washed in 2× SSC, 0.1% SDS, twice at room temperature; once at 65°C; then once in 0.2× SSC, 0.1% SDS at 65°C. Autoradiography was performed and the signal quantified using scanning densitometry. Cloned DNA standards were used. The lower limit of detection was 4 pg/ml.

PCR

DNA was extracted from 50 μl serum as previously described [Carmen et al., 1991]. DNA was ethanol precipitated and resuspended in 20 μl water; 5 μl of this were used in a nested PCR reaction. The primers used were to the core region [outer: 5′ GCGCTGCAGGAGTTGGGGGAGGAGATTA 3′ (positions 1735–1755) and 5′ TTGATAAGATAGGGGCATTTG 3′ (2304–2324) and inner: 5′ GCGCTGCAGGAGGCTGTAGG—CATAAAT 3′ (1775–1794) and 5′ GCGAAGCTTAGATCTCTGGATGCTGGA-3′ (2134–2152) and also to the surface region (outer: 5′ GCGCTGCAGCTATGCCTC—ATCTTC 3′ (positions 418–433) and 5′ GCGAAGCTTGCTGTACAGACTTGG 3′ (761–776) and inner 5′ GCGCTGCAGCAAGGTATGTTGCCCG 3′ (455–470) and 5′ GCGAAGCTTCATCATCCATATAGC 3′ (734–748)]. Ten microliters of each first-round reaction was used for the nested reaction, and 10 μl of the second-round product were run on a 2% agarose gel and visualised using ethidium bromide staining. Negative controls included an extraction control of a patient seronegative for HBsAg and water as a control for the PCR. A positive control from a patient with high-titre HBV DNA as measured by dot blot hybridisation was included.

Serology

HBsAg, HBeAg, and anti-HBe were determined by HBeAg/anti-HBe and HBsAg commercial assays (Murex Diagnostics).

HBcAg Staining

Formalin-fixed paraffin- embedded liver biopsy sections were stained for HBcAg with rabbit polyclonal anti-HBc serum (Dako) by a two-layer immunoperoxidase technique.

Statistical Analysis

Statistical analysis was performed using Student's t test or Fisher's exact test when appropriate.

RESULTS

Overall 37 of 71 (52%) patients were positive for HBV DNA by the bDNA assay (Table I). Twenty-one of twenty-eight (75%) HBeAg-positive patients and 14 of 36 (39%) anti-HBe-positive patients were positive ($P = 0.09$). The mean HBV DNA levels were significantly higher in the HBeAg-positive group than in the anti-HBe-positive group (8,317 ± 8,903 pg/ml vs. 123.7 ± 173; t = 4.21, $P < 0.01$). Serum transaminases were similar in HBV DNA-positive and negative patients; 16 of 38 (42%) HBV DNA-positive patients had normal serum aminotransferase concentrations compared to 19 of 34 HBV DNA-negative patients (56%). We found no correlation between serum ALT and DNA concentrations in all 71 patients or specifically within the HBeAg positive or anti-HBe-positive groups. Fifteen patients were anti-HDV positive. Eight were positive for HBV DNA by the bDNA assay (mean level 716.4 ± 1808 pg/ml), four of them being HBeAg positive and four anti-HBe positive.

Liver Histology

When patients who had received successful anti-viral therapy in the interval between liver biopsy and serum HBV DNA testing were excluded from the analysis, there was no significant difference in the histological findings in bDNA-positive or bDNA-negative patients (Table I). bDNA-positive patients were, however, significantly more likely to have positive staining for HBcAg ($P < 0.05$).

Comparison Between Dot Blot Assay and bDNA Assay

Sixty-four serum samples tested by bDNA were also assessed for HBV DNA by dot blot hybridisation. Sixteen (25%) were positive by both assays and 20 (31%) by the bDNA assay alone. The remaining 28 (44%) were negative by both assays. None were positive by dot blot alone. This indicates a greater sensitivity of the bDNA assay. The mean HBV DNA level as assessed by the bDNA assay was significantly higher for dot blot-positive patients than for dot blot-negative patients (11,942 ± 8,654 pg/ml vs. 41.5 ± 206.0; t = 5.67; $P < 0.01$ Fig. 1). Among the 20 patients with measurable DNA by the bDNA assay but not by dot blot, 8 were HBeAg positive, 12 had raised serum transaminases, and, among the 14 of the 20 who had liver biopsies, 9 had active hepatitis.

Comparison Between PCR and bDNA Assay

Twenty-five bDNA-negative patients were available for testing for HBV DNA by PCR. Sixteen (64%) were PCR positive using primer for either the surface or the core regions of the HBV genome. Two of the sixteen were HBeAg positive but subsequently lost HBeAg. The remainder were HBeAg negative.

DISCUSSION

We have assessed the sensitivity and clinical use of a new signal amplification assay for quantitating HBV DNA. The Chiron signal amplification assay was relatively simple to use. The use of signal, as opposed to DNA amplification, and the use of nine probes to capture the HBV DNA and 39 target probes to bind the amplifier reduce the chances of both false-positive and false-negative results. Furthermore, the provision of quantitative standards makes the assay more reproducible and diminishes the chances of interlaboratory variation.

The findings indicate that the bDNA assay was more sensitive than the in-house dot blot assay for the quantification of HBV DNA; the assay has been found previously to be approximately 100 times more sensitive than the Abbot (liquid hybridisation) assay [Valinluck et al., 1993]. Serological criteria of HBV are inadequate per se to assess and treat patients with chronic hepatitis B, and direct quantitation of serum HBV DNA concentrations is also frequently required. Our data suggest that at least one third of the anti-HBe positive cohort had evidence of replicative hepatitis B infection and could be considered for antiviral therapy.

The assay also proved useful in indicating cessation of replication after antiviral therapy; for example, we identified six HBeAg-positive HBV DNA-negative patients after antiviral therapy. Five of these patients subsequently lost HBeAg, so our data are comparable to those obtained using liquid hybridisation to measure HBV DNA [Perrillo et al., 1993].

PCR can detect 10 HBV particles per milliliter of serum and is therefore more than 10^4 times more sensitive than the signal amplification assay. Its unique sensitivity, however, means that PCR does not differentiate patients who may benefit from antiviral therapy. In our series, only 5 of the 16 patients who were bDNA negative and PCR positive had raised serum transaminases, and, among the four of these five patients who had liver biopsies, only one had active hepatitis histologically and would have been considered a potential candidate for interferon-α or other antiviral therapy. These data indicate the greater clinical selectivity of the bDNA assay in comparison to PCR.

The relationship between replicative hepatitis B infection, hepatic necrosis, and the pathogenesis of chronic hepatitis B is complex. Immunologically mediated injury is important, and many patients with high levels of viraemia will not have raised serum aminotransferases. None

TABLE I. Comparison of HBV DNA-Positive and -Negative Patients as Measured by the Branched Chain DNA Assay

Characteristics	DNA positive	DNA negative	P
Total patients	37	34	ns
Clinical features			
Males	28	24	ns
Females	9	10	ns
Median age (years)	37.5	40	ns
Median duration of infection (years)	32	27	ns
Mean ALT (IU/ml)	64.9 ± 54.7	66 ± 62	ns
Serology			
HBeAg⁺/anti-HBe⁻	21	7	0.09
HBeAg⁻/anti-HBe⁺	14	22	
HBeAg⁺/anti-HBe⁺	1	3	ns
HBeAg⁻/anti-HBe⁻	1[a]	2	ns
Anti-HDV⁺	8	7	ns
Liver histology*			
Mild hepatitis	4	1	
Active hepatitis	17	10	ns
Cirrhosis	3	3	
Positive staining for HBsAg	14	2	ns
Positive staining for HBcAg	9	0	<0.05
Dot blot positive	16	0	<0.03
Dot blot negative	20	28	

*Liver biopsy data exclude patients who had received successful antiviral therapy between the time of the biopsy and measurement of serum HBV DNA.
[a]HIV positive.

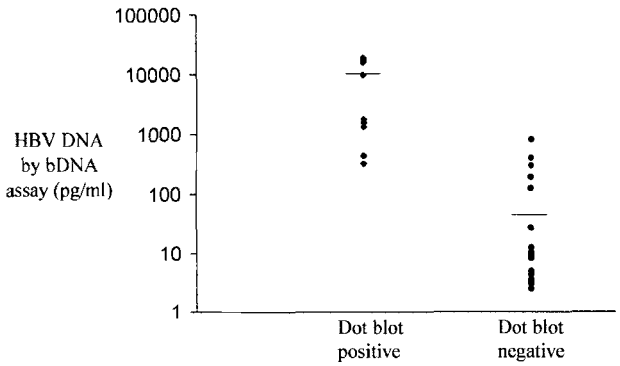

Fig. 1. Comparison of mean HBV DNA levels as measured by the bDNA (branched chain DNA) assay in dot blot-positive and dot blot-negative cases. Cases negative for HBV DNA by the bDNA assay are not shown, all were dot blot negative.

of the assays we used for measurement of serum HBV DNA, in either HBeAg-positive or anti-HBe-positive patients, correlated with serum ALT. In our series, 12 of the 20 dot blot-negative, bDNA-positive patients had raised serum transaminases. There was some correlation between a positive bDNA test and active histological hepatitis; 9 of 14 patients who had liver biopsies had active hepatitis, indicating that the assay might identify a clinically relevant population. Active hepatitis was also identified in bDNA-negative patients, however.

Nucleoside analogue therapy with agents such as famciclovir and lamivudine will be used increasingly. The criteria for selection of patients for antiviral therapy might have to be broadened to include all patients with active viral replication as measured by serum DNA testing. In this circumstance, the use of a more sensitive assay for the detection of serum HBV DNA, but not PCR, might allow more appropriate patient selection and subsequent monitoring. The assay should provide a useful, standardised tool for the management of such patients and monitoring of therapy. However, the limitations of the assay are demonstrated by the fact that, in our analysis, 10 patients with histologically active hepatitis were bDNA negative, indicating that not all potential candidates for antiviral therapy are identified. Our data suggest that the bDNA assay provides some additional sensitivity for HBV replication and some degree of discrimination of patients suitable for antiviral therapy.

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